Gene Regulation in Human Airway Epithelial Cells in Response to Pneumolysin

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Abstract

Pore-forming toxins introduce pores in cell membranes, lead to cell death, and cause overall tissue damage. Among these toxins is pneumolysin, a cholesterol-dependent toxin produced by *Streptococcus pneumoniae*, which is involved in causing pneumonia as well as other health problems. Due to the lack of novel antibiotics, there is a need for new antimicrobial agents. It is plausible that protecting cells against pneumolysin attack could lead to resistance to *S. pneumoniae* infection. My research project is to establish a panel of genes that are transcriptionally regulated upon pneumolysin treatment in human airway epithelial cells. Using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) technique to optimize the time course and dose response of pneumolysin treatment in the cells, we could then further use this readout to screen for drugs that could lead to the change in pneumolysin-response gene expression and further protect the cells against pneumolysin.

Introduction

Because drug-resistant bacteria render antibiotics useless, there is a desperate need to develop novel antibiotics against these bacteria (1). One such bacterium is *Streptococcus pneumonia*, a type of bacterium that secretes a cholesterol-dependent cytolysin (CDC) called pneumolysin (PLY), which contributes to pneumonia (2). CDCs are cholesterol-dependent toxins secreted by many gram-positive bacteria that induce large pores in cholesterol-containing membranes (2). Two models have been suggested to describe the mechanism of how CDCs insert large pores into the membrane (2). Of the two models, the more favored model describes CDCs as soluble monomeric proteins that bind to the cholesterol of the cell membrane, aggregate into an insoluble oligomer, which subsequently inserts itself through the membrane as a transmembrane beta-barrel complex (3). PLY elicits immune response from cells to up-regulate certain genes for immunity, such as proinflammatory cytokines like interleukin-8 (IL-8) (4), and inflammasome activation of IL-1β and IL-18 via caspase-1 activation (5). Although several genes have been shown to be induced in response to PLY, these experiments were done in different cell types and not specifically in human airway epithelial cells. Since the human airway epithelial cell is the main target tissue of *S. pneumoniae* infection, this research paper focuses on determining a panel of up- and down-regulated genes specifically in the human bronchial epithelial cell line, HBE-1, and primary human tracheal epithelial cells in response to PLY. We aim to set a reliable transcriptional readout to find novel regulatory pathways involved in the airway epithelial cells’ defense against PLY so that we can screen for candidate drugs that could help cell survival in response to PLY. To determine a panel of possible genes that are either up- or down-regulated, we treated cells with varying concentrations of PLY (ng/mL), extracted RNA, produced cDNA, and performed qRT-PCR to quantitatively determine regulation of the following genes: spliced form of xbp-1, CHOP, Cpn60,
ClpP, IL-8, Interferon receptor (INFAR), IL-1β, IL-18, GABARAP, GABARAP-L1, GABARAP-L2, WIPI-1, WIPI-2, ATG-12 and HMGCR. The choice of genes was based on previous reports and our previous findings involving several protective pathways against pore-forming toxins.

**Methods**

**Toxin Treatment**

Human airway epithelial cells (HBE-1 and primary cells) were seeded into 6 well plates at a density of roughly 500,000 cells per well. After overnight incubation, cells were treated with vehicle control or pneumolysin at various concentrations for 4 hours. HBE-1 cells were treated with 0, 50, 100, 200, and 400 ng/mL of PLY. Primary cells obtained from two patients – Patient #66799 and #63858 – were treated with 0, 100, 200, and 400 ng/mL of PLY.

**RNA Extraction**

Cells were rinsed twice with cold PBS and lysed with Trizol. RNA was stored in 30µL - 50µL of RNase free water. RNA concentration was determined using a Nanodrop Spectrophotometer and ND1000 V3.30 program with 1.5 µL of RNA solution.

**Reverse Transcription**

For production of cDNA, 2 µg of RNA were mixed with RNase free water, oligonucleotides, 5 x RT buffer, RNase inhibitor (40U/µL), 10 mM dNTP, and Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV). The reaction was performed in a thermocycler for reverse transcription to occur.

**qRT-PCR**

For qRT-PCR, cDNA and a final master mix containing RNase free water, SYBR® Green master mix, and primers were analyzed by the ABI ViiA7 system for quantitation of the transcriptional levels of the genes of interest.

**Statistics**

The significance of the group differences was compared by one-way ANOVA with Dunnet’s post-test comparing all the treatment groups with the controls. P< 0.05 is considered significant.

**Results and Conclusions**

![Fig. 1a](image1.png)

![Fig. 1b](image2.png)
Fig. 1m

Fig. 1n

Fig. 1o

Fig. 1p

Fig. 1q

Fig. 1r

Fig. 1s

Fig. 1t

Fig. 1u

Fig. 1v
Figure 1. Graphs showing gene expression levels of spliced form of xbp-1 (Figs.1a, b), CHOP (Figs.1c, d), Cpn60 (Figs.1e, f), ClpP (Figs.1g, h), IL-8 (Figs.1i, j), INFAR (Figs.1k, l), IL-1β (Figs.1m, n), IL-18 (Figs.1o, p), GABARAP (Figs.1q, r), GABARAP-L1 (Figs.1s, t), GABARAP-L2 (Figs.1u, v), WIPI-1 (Figs.1w, x), WIPI-2 (Figs.1y, z), ATG-12 (Figs.1aa, bb), and HMGCR (Figs.1cc, dd) in relation to each of their own respective control groups in HBE cells and primary cells from two patients (Patient #66799 and Patient #63858). Figs.1a, c, e, g, i, k, m, o, q, s, u, w, y, aa, and cc show HBE cells treated with 0, 50, 100, 200, and 400 ng/mL of PLY with the treatment of 0 ng/mL of PLY as the control group. Figs.1b, d, f, h, j, l, n, p, r, t, v, x, z, bb, and dd show primary cells treated with 0, 100, 200, and 400 ng/mL of PLY with the treatment of 0 ng/mL of PLY as the control group.
ER-UPR

Xbp-1 spliced is involved in stress response in the Endoplasmic Reticulum unfolded protein response (ER-UPR) (13). There are three pathways that induce C/EBP homologous protein, and xbp-1 spliced is a transcription factor in one of three pathways that induces C/EBP homologous protein (CHOP) (12). In Figure 1a, HBE-1 cells display a significant decrease in xbp-1 spliced form expression in response to PLY, which suggests that PLY causes a decrease of xbp-1 activation. In contrast to HBE-1 cells, the primary cells of both patients display different trends in xbp-1 activation. The variance in gene response shows that more primary cells should be tested with the xbp-1 spliced form so that we could gain a more accurate idea of how the xbp-1 pathway is regulated in human primary cells. Statistical analysis showed that the decreased levels in the xbp-1 pathway are significant enough to conclude that the xbp-1 pathway is down-regulated in response to PLY.

C/EBP homologous protein (CHOP) is a transcription factor that plays a role in response to growth arrest or DNA damage (6), and is produced through three different pathways in the ER-UPR pathway. There is evidence that CHOP is linked to apoptosis (6). In Figure 1c, the increasing trend in CHOP expression in HBE-1 cells suggests that PLY causes up-regulation of CHOP. Figure 1d shows varying levels of CHOP induction in primary cells. Although there is variance in primary cell CHOP expression levels, more primary cells should be screened for CHOP to obtain a clearer picture of CHOP induction. However, because statistical analysis showed that CHOP is not significantly induced in response to PLY, we can only conclude at the moment that we see an increasing trend in CHOP.

Down-regulation of xbp-1 spliced indicates that the increasing trend seen in CHOP regulation occurs through another ER-UPR pathway – either PERK or ATF-6. Furthermore, because PLY forms large pores in the cell membrane, it is plausible that the severe membrane breach leads to the introduction of foreign material into the cell, causing heavy cell damage, the leaking of cellular nutrients out of the cell, and possible activation of apoptosis. Down-regulation of xbp-1 splice and increased regulation of CHOP could mean that the cells favor apoptosis as a response.

MT-UPR

Cpn60 is a stress-induced chaperone that plays a role in the mitochondrial unfolded protein response (MT-UPR) (6). ClpP is a gene that encodes for the catalytic subunit of mitochondrial protease and is induced when the mitochondria is under stress (6). Figure 1e and f display Cpn60 regulation in HBE-1 cells and primary cells, respectively, and Figure 1g and h, for ClpP in HBE-1 cells and primary cells, respectively. Neither HBE-1 cells nor primary cells display a significant increase or decrease in Cpn60 and ClpP expression. Statistical analysis confirms that there is no significant up- or down-regulation. This suggests that Cpn60 and ClpP are not induced in response to PLY and that the MT-UPR pathway is not affected by PLY. It is possible that, although the cell is experiencing damage, the mitochondria were kept well intact during PLY treatment, which is why activation for genes in response to MT-UPR is not needed. One reason why the MT-UPR pathway is not induced is because the mitochondria lacks cholesterol around its inner membrane, so PLY cannot induce large pores to penetrate and damage components within the inner membrane of the mitochondria.
**Inflammatory Pathway**

IL-8 is a chemokine that plays a role in intercellular communication (14) and is part of the inflammatory pathway. Figure 1i shows HBE-1 cells having a slight increase in IL-8 expression, while Figure 1j shows primary cells with a huge induction of IL-8 expression, suggesting up-regulation in IL-8 in response to PLY treatment. Even with the huge induction in primary cells, the trend in Figure 1j is decreasing; this is because, as PLY concentration increased, more cells died, lowering cell count and thus lowering levels of IL-8 production. Statistical analysis suggests that IL-8 is significantly induced by PLY. Therefore, we can conclude that IL-8 is one pathway induced by PLY in human airway epithelial cells.

Interferon receptor (INFAR) is a receptor gene for cytokines that defend against viral and parasitic attacks (19). Figures 1k and l display INFAR response levels in HBE-1 cells and in primary cells, respectively. Both figures display very small variance in expression levels compared with the control group. Statistical analysis showed that there is no significant difference between the treated HBE-1 cells and primary cells, when compared with their respective control groups. We can therefore conclude that INFAR gene expression is not induced by PLY.

**Inflammasome Pathway**

Inflammasomes are activated by caspase-1, which promotes cell membrane repair (5). One inflammasome readout that we tested for gene regulation is IL-1β, which activates adhesion molecules that are needed to send leukocytes to inflammation areas (4). Another inflammasome readout, called IL-18, is a cytokine that helps produce T cells for immune response (16). IL-18 is also a proinflammatory cytokine that elicits an inflammatory response, but high levels of IL-18 resulting in damage to the host cell is the hallmark of inflammasome activation (15). Figure 1m and n, which depict HBE-1 cells and primary cells, respectively, display no induction in IL-1β expression. Figure 1o and p, which depict HBE-1 cells and primary cells, respectively, display no induction in IL-18 expression, suggesting that there is no induction of the inflammasome pathway. One reason that the cell may induce the inflammatory pathway instead of the inflammasome pathway is that the inflammatory pathway provides a temporary immune response against PLY, whereas the inflammasome pathway provides a more long-term immune response. The cell therefore expends less of its resources on a short-term defense response compared with a longer-term response against PLY.

**Autophagy**

Autophagy is a cellular response against nutrient and/or growth-factor stress in which the cells direct toxin, misfolded proteins, and pathogens at lysosomes, which degrade as a result. Autophagy is also linked to the apoptosis pathway (9). GABARAP, GABARAP-L1, GABARAP-L2, WIPI-1, WIPI-2, and ATG-12 are genes related to autophagy (9, 17). Figures 1q, r, s, t, u, v, w, x, y, z, aa, and bb display the response levels for GABARAP, GABARAP-L1, GABARAP-L2, WIPI-1, WIPI-2, and ATG-12 genes, respectively. Of all the autophagy genes tested, only GABARAP-L1 shows a slight increase in expression in HBE-1 cells and Patient #66799 primary cells (Figures 1s and t). This could indicate GABARAP-L1 was up-regulated in response to PLY. According to unpublished data on autophagy response in Western blots, autophagy genes are up-regulated in response to PLY. Further investigation
in other autophagy genes should be undertaken to determine which particular autophagy genes are up-regulated in human airway epithelial cells.

**HMGCR**

HMG-CoA reductase (HMGCR) is an enzyme that contributes to the production of cholesterol in the cell (18). It is thought that inhibition of production of cholesterol increases cell survival against PLY, as PLY is cholesterol dependent. Figures 1cc and dd display HBE-1 cells and primary cells, respectively, exhibiting no significant change in HMGCR expression levels compared with their respective control groups. One possible reason that the cell would not down-regulate the production of cholesterol and thus inhibit PLY from forming large pores in the membrane is that, as cholesterol is important to membrane biosynthesis (18), the cell could not dispose of cholesterol enough to compensate for the inability of PLY to bind.

PLY elicits down-regulation in the xbp-1 pathway in ER-UPR response and shows an increasing trend for CHOP regulation. The inflammatory (IL-8) pathway is up-regulated and there is evidence that the autophagy pathway is induced. MT-UPR, inflammasome, and HMGCR are not induced at the transcriptional level. In future investigation, we plan to check the PERK and ATF-6 gene regulation in the ER-UPR pathway, since they are both candidates for inducing CHOP gene expression. More samples should be tested to more accurately determine which autophagy genes are induced by PLY. Because of the variance in gene expression levels in primary cells, testing for gene expression with additional primary cells from different patients will give a more accurate picture of which genes are truly induced in the human airway epithelial cells in response to PLY. Our long-term goal is to use induced genes as markers to screen for future potential candidates for drugs that fight pneumolysin and, ultimately, resist pneumonia.

**Acknowledgements**

This work was supported by Reen Wu at the University of California, Davis, Department of Anatomy, Physiology, and Cell Biology. The author thanks Reen Wu and Cheng-Yuan Kao for comments on this manuscript; Cheng-Yuan Kao for guidance on experimental procedures, figures, and statistical analysis; and other lab members who supported this project.

**References**


